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Model systems for the study of the role of PADPRP in essential biological processes

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Summary — The nuclear enzyme poly(ADP-ribose) polymerase (PADPRP) is implicated in a number of cellular processes, including DNA repair, replication, and differentiation. We have been using several model systems to examine these potential roles of PADPRP. A human keratinocyte model system has been developed in which stable lines of epidermal cells contain an inducible construct harboring the antisense cDNA to PADPRP. When PADPRP antisense RNA is induced in culture, endogenous PADPRP mRNA, protein, and enzymatic activity is lowered, and the pattern of poly(ADP) ribosylation in response to alkylating agents is altered. When keratinocyte clones containing the antisense construct or empty vector alone were grafted onto nude mice, they formed histologically normal human skin. The PADPRP antisense construct was also inducible *in vivo* by the topical application of dexamethasone to the reconstituted epidermis, as determined by *in situ* hybridization. In addition, poly(ADP-ribose) polymer could be induced and detected *in vivo* following the topical application of a DNA alkylating agent to the grafted transfected skin layers. Thus, a model system has been developed in which the levels of PADPRP can be selectively manipulated in human keratinocytes in cell culture, and potentially in reconstituted epidermis as these keratinocyte lines can be grafted to nude mice, whereupon they form a histologically and immunocytochemically normal human epidermis. Another system that we have been utilizing to examine the role of PADPRP in proliferation and differentiation is stable lines of mouse preadipocytes that contain an inducible antisense PADPRP RNA. Similar to the keratinocyte system, these cells can inducibly express antisense PADPRP RNA, and subsequently lower levels of endogenous PADPRP. In this system, the normal differentiation to adipocytes is blocked by the lowering of endogenous PADPRP, apparently resulting from inhibition of replication immediately preceding terminal differentiation. This inhibition in turn may stem from the requirement for the physical association between PADPRP and polymerase alpha during the S phase of the cell cycle. These systems will be useful tools to study the role of PADPRP in essential biological processes.

differentiation / keratinocytes / DNA repair / grafting / dexamethasone

Introduction

Poly(ADP-ribose) polymerase (PADPRP) is a nuclear enzyme that has been implicated in a number of biological processes, including DNA repair, cellular differentiation and transformation, sister chromatid exchange, and gene rearrangements and transpositions [1–3]. Although relatively little is known about how PADPRP performs these functions, its structure and biochemical properties have been studied in great detail. PADPRP contains an amino-terminal zinc finger DNA-binding domain, a central automodification domain, and a carboxy-terminal catalytic domain. PADPRP binds to and is activated by the free ends of DNA. The active enzyme thus catalyzes the poly(ADP-ribosyl)ation of select nuclear proteins in the vicinity of the damaged DNA, using nuclear NAD as a substrate.

PADPRP can be activated by a number of agents, including chemical carcinogens, as well as by ultraviolet and ionizing radiation. Thus, PADPRP is likely to play an important role in the repair of DNA within the epidermis. The localization of PADPRP to the lower layers of the epidermis including the proliferating cells, which are susceptible to DNA damage, is consistent with this notion [4]. The role of PADPRP in mediating the biological response to DNA strand breaks in the epidermis is supported by a number of studies. These experiments show an increase in PADPRP activity may be responsible for the blistering response of skin exposed to sulfur mustards, and possibly to other DNA strand breaking agents [5–10]. Numerous studies have also demonstrated a role for PADPRP in cellular proliferation and differentiation [11, 12].

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The link between the roles of PADPRP in these varied biological processes, as well as the relationship between its enzymatic activity and ultimate function, is largely a matter of conjecture. *In vitro* systems have been utilized to elucidate the connection between the DNA-binding and ADP-ribose polymerizing activity; and the biological activities of PADPRP. It was recently demonstrated [13] that unmodified PADPRP binds to damaged DNA *in vitro* and inhibits DNA repair, except in the presence of NAD. This suggested that PADPRP alternates between an unmodified form, which blocks the ends of DNA, and a modified form, which is released from DNA, thereby allowing access of repair enzymes [13]. We confirmed this model of PADPRP cycling using bacterially-expressed deletion mutants of PADPRP [14]. Our data with the *in vitro* assay of DNA repair showed that mutants containing an intact DNA-binding domain show an NAD-suppressible inhibition of DNA repair when added to a PADPRP-depleted HeLa cell extract. However, deletions in the automodification domain or the NAD-binding domain prevented alleviation of the inhibition exerted by these mutants by NAD. Most other studies supporting a function for PADPRP in these processes have relied on the use of chemical inhibitors of the enzyme, many of which (*eg* thymidine, 3-aminobenzamide, and benzamide) lack specificity [15].

Results and discussion

In order to study the function of PADPRP, and to avoid the non-specific effects of chemical inhibitors, we recently established several model systems to selectively lower levels of PADPRP by the use of constructs that express antisense transcripts to the PADPRP cDNA. When these constructs are induced, levels of PADPRP mRNA are reduced as well. Furthermore, biological function in these cells was altered. In HeLa cells, DNA repair in response to strand breaking agents was delayed, active gene repair was reduced, and there was increased amplification of genes coding for selectable markers [16–18].

We have therefore used this antisense model system to examine the role of PADPRP in differentiation. Previously, in culture systems, differentiation has been shown to be associated with alterations in the expression of PADPRP, and inhibition of PADPRP can modulate the program of terminal differentiation [19–21]. To further examine the role of PADPRP in differentiation and DNA repair, we were interested in developing a system to selectively lower the levels of PADPRP in cultured human keratinocytes, and in reconstituted epidermis. The involvement of PADPRP in differentiation in the epidermis was also of particular interest to us, since epidermal differentiation is marked by a now well-defined sequence of biochemical and physiological changes that allow the progression from dividing basal cells to mature cornified envelopes. Furthermore, perturbations in this process can lead to a variety of skin lesions, including squamous cell and basal cell carcinoma.

We generated stable lines of keratinocytes that contain inducible antisense RNA to PADPRP, using immortalized, but not transformed cells, as determined by the graft system (below). For this purpose, we used cells immortalized with the E6 and E7 genes of HPV 18, which formed a histologically and immunocytochemically normal skin. These cells were transfected with a plasmid containing the human PADPRP in the antisense orientation under the control of the MMTV promoter (fig 1), and stable clones were isolated. PCR analysis revealed that the PADPRP antisense construct was stably integrated in most clones. Three stable lines (AS-1, AS-2, and AS-3) containing the antisense construct were chosen for further analysis, along with control cells transfected with the vector alone.

A ribonuclease protection assay (RPA) was employed to determine the levels of endogenous PADPRP RNA. Control or antisense cells were induced with dexamethasone for 0, 24 or 48 h. Total RNA was then isolated and analyzed by the RPA.

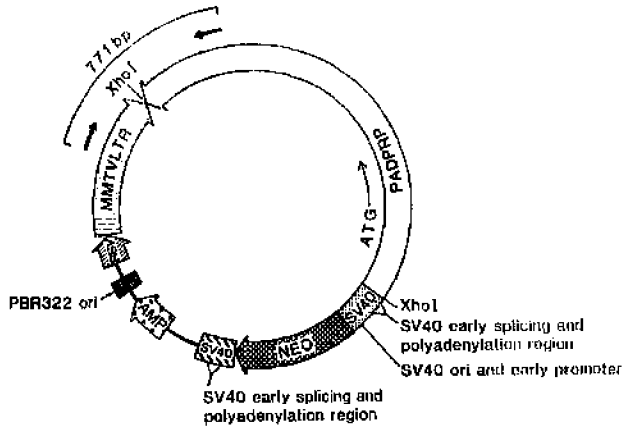

Fig 1. Structure of pMAMneo containing PADPRP antisense cDNA, and location of PCR amplimers. The expression vector contains the glucocorticoid inducible MMTV promoter ligated to an inverted orientation of human PADPRP cDNA comprising the entire PADPRP untranslated and translated regions. The PADPRP sequence is flanked downstream by the SV40 early splicing and polyadenylation region and the neomycin (neo) and ampicillin (amp) resistance genes. The entire cloned plasmid is 12.2 kb. Amplimers used for PCR analysis are shown as black arrows.

using a 588 bp probe homologous to the 3' region of the endogenous PADPRP mRNA. In all cells, a prominent 588/578 bp doublet was protected at time 0, which indicates the presence of two different sized PADPRP transcripts in these cells. In control cells, there was no reduction in the endogenous levels of PADPRP RNA. Additionally, the levels of a 220 bp ribosomal protein mRNA fragment, used as a control, were not altered. However, in antisense cells, the PADPRP mRNA doublet was almost undetectable in all antisense clones tested 48 h after induction, even though there was no reduction in the level of the 220 bp control probe, indicating the selective elimination of the endogenous PADPRP transcripts.

Using an enzyme assay, the endogenous levels of active PADPRP protein were then measured. Control cells and non-induced antisense cells demonstrated an equivalent amount of endogenous PADPRP activity. However, when antisense cells were induced by dexamethasone, the level of PADPRP activity was reduced by 80% within 48 h.

To determine if steady-state levels of endogenous PADPRP protein were reduced, antisense-containing keratinocytes were treated with 1 μM dexamethasone for either 24, 48, 72, or 96 h, and extracts containing equal amounts of protein separated by SDS-PAGE. Figure 2 shows the results of the immunoblotting experiment in which filters were probed with polyclonal antiserum specific for human PADPRP. Visualization of the membranes using ECL revealed that the levels of PADPRP were significantly reduced in cells containing antisense by 48 h post-treatment. In contrast, control cells showed no reduction in the level of PADPRP after induction by dexamethasone for more than 72 h.

We examined whether there was any alteration in the response of these keratinocytes to DNA strand-breaking reagents, by staining cells simultaneously with PADPRP and polymer antibody. Cells were treated with MNNG to induce DNA strand breaks, either with or without induction of antisense RNA. Double immunofluorescence of control cells revealed abundant levels of PADPRP under all conditions tested. Staining for poly(ADP-ribose) polymer revealed that polymer was only formed in the presence of MNNG. When cells containing the PADPRP antisense construct are induced with dexamethasone prior to MNNG treatment, almost no PADPRP can be detected in individual cell nuclei, and the PADPRP that is present is localized to distinct focal regions within the nucleus. These same PADPRP-positive focal areas are also positive for poly(ADP-ribose). However, the amount of polymer is drastically reduced compared to both control cells and dexamethasone-uninduced antisense cells.

Accordingly, the transfected keratinocytes appeared to possess those biochemical properties which would be desirable to ascertain the function of this enzyme in skin layers. Both control and antisense keratinocytes formed an essentially normal epidermis as discerned by histological analysis. Additionally, the reconstituted human epidermis demonstrates a similar staining pattern for human keratins 10 and 14 to that of normal human epidermis. Thus, the normal pattern of histological and immunocytochemical pattern of differen-

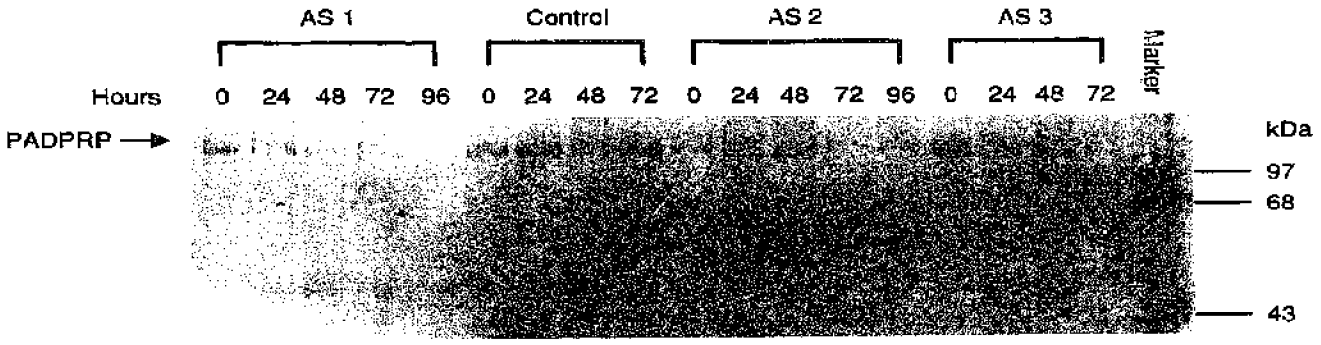

Fig 2. Effect of dexamethasone induction of antisense RNA on cellular PADPRP content as determined by immunoblotting. Control and antisense-containing cell clones (AS1, AS2, and AS3) were grown in the presence or absence of dexamethasone (1 μM). At the times indicated, cells were collected and washed with phosphate-buffered saline, and their protein concentration was determined. Equal amounts of total cellular protein (20 μg) were subjected to electrophoresis on duplicate SDS polyacrylamide gels. Enzyme-linked immunoblotting with rabbit antibodies to human PADPRP was performed. The 116 kDa arrow indicates the position of human PADPRP. The disperse staining for PADPRP may indicate the activation and automodification of the nuclear enzyme in these cells under the culture conditions used.

tiation was observed in the reconstituted grafted epidermis.

We then determined whether the antisense RNA could be induced in cells containing the antisense constructs, and then used to form a reconstituted epidermis in the graft system. Grafting was performed as described above, and then the animals were treated topically with 75 μg of dexamethasone in acetone at the graft site. *In situ* hybridization for the presence of antisense transcripts within the skin, revealed that dexamethasone treatment induced antisense transcripts within the epidermis (fig 3).

Finally, we set out to determine whether poly(ADP-ribose) polymer formation, *via* its post-translational modification of nuclear proteins, could be induced in the grafted epidermis in response to DNA strand-breaking agents. Skin grafts treated with topical alkylating agents formed polymer as detected by immunocytochemistry.

Several features of this engineered epidermis make it extremely useful for further long-term studies of PADPRP or other enzymes within the epidermis. In addition to the aforementioned ability of these cells to form a morphologically and immunocytochemically normal epidermis, we have been able to immediately monitor the level of DNA damage within the epidermis *in vivo* by the use of monospecific antibodies directed against the poly(ADP-ribose) polymer. Thus, we can manipulate antisense, and measure the immediate response to DNA damage, as well as the long-term response, such as the mutation of genes critical for epidermal differentiation or transformation.

The 3T3-L1 preadipocyte cell line also represents a useful tool for studying mechanisms of cellular differentiation [22–24]. When appropriately induced by a defined hormonal treatment (insulin, dexamethasone, and methylisobutylxanthine), 3T3-L1 preadipocytes differentiate in culture into cells that possess morphological and biochemical characteristics of adipocytes [23]. Depending upon the assay used, both decreases as well as increases in PADPRP activity have been reported as one of the earliest events during differentiation in these cells [19, 21]. Accordingly, with the antisense expression conditions for HeLa cells as a guide, we have analyzed how altering the concentration of PADPRP, by antisense RNA expression, may

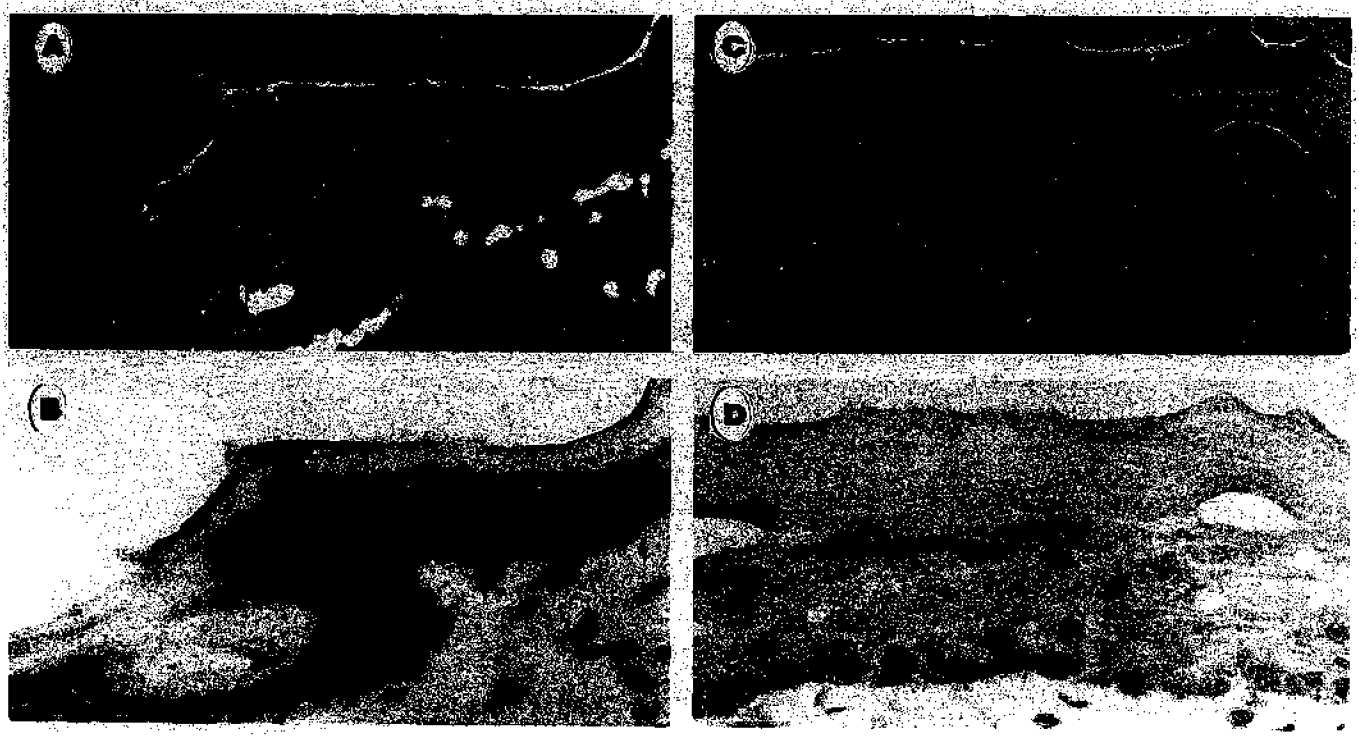

Fig 3. Induction of PADPRP antisense transcripts *in vivo* by dexamethasone. Cells transfected with antisense construct (**A** and **B**) or vector alone (**C** and **D**) were grafted onto nude mice. After 3 weeks, mice were treated topically with dexamethasone or acetone for 24 h. Frozen sections were derived and analyzed for antisense transcripts using a riboprobe complementary to antisense transcripts.

affect the onset and progression of cellular morphological changes in 3T3-L1 preadipocytes during differentiation. Both PADPRP protein and activity show a transient, marked increase on exposure of cells to inducers of differentiation. The increase in PADPRP expression is prevented by antisense RNA synthesis. Under these conditions, both cellular proliferation and differentiation did not occur.

We have thus shown that in 3T3 L1 preadipocyte mouse cells, the induction of antisense RNA directed against the 1.1 kb 5'-most region of the mouse PADPRP cDNA results in the inhibition of differentiation of cells into mature adipocytes in response to the differentiating agents insulin, dexamethasone, and methylisobutylxanthine. It appears that PADPRP is important in the terminal replicative cycles prior to differentiation [25]. In cultured keratinocytes, a similar phenomenon of a near-synchronous wave of replication prior to differentiation is observed. Various models, all of which involve chromatin restructuring, have been proposed to explain the necessity for DNA replication prior to differentiation [26]. Implicit in many of these models is the repositioning or alteration of nucleosomes which may result in the activation or inhibition of specific genes and may involve various nuclear protein modifications, including poly(ADP-ribosyl)ation. In this regard, poly(ADP-ribosyl)ation may aid in either relaxation [27] or condensation of chromosomal proteins around the replicating regions of chromatin [28]. Whether PADPRP function is essential to the terminal differentiation process in keratinocytes and in preadipocytes can now be tested directly with the *in situ* and cell culture systems developed in these studies. Taken together, these data provide the basis for future work that would utilize induction of the antisense constructs by dexamethasone to reduce the levels of PADPRP protein and correspondingly lower PADPRP activity in the human keratinocytes grafted to mice, and in the 3T3 L1 differentiation system.

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